Appendix 1.

Methods

Statistics

Q-Q plots revealed that the [U] data was not compatible with a Gaussian distribution. To test the difference in median [U] between wild type patients and *DPYD*-variant carriers the Wilcoxon rank sum test was used. The reported difference and the corresponding confidence intervals are reported in the relevant figures. The overall difference in median [U] across all tested *DPYD*-variants, and hospital laboratories were tested using the Kruskal-Wallis test. All statistical analyses were performed using R version 4.0.2

(R Core Team. R: A Language and Environment for Statistical Computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2020. Available from: https://www.R-project.org/)

DPYD-genotype analysis

Four laboratories (Rigshospitalet, Odense University Hospital, Aarhus University Hospital, and Zealand University Hospital from April 2021) used the LAMP (loop-mediated isothermal amplification) technology. At Zealand University Hospital, Sanger sequencing was used for some of the first samples until April 2021. At University Hospital of Southern Denmark, Lillebaelt Hospital, Real-time PCR was performed using four commercial TaqMan assays. At Aalborg University Hospital, the variants were identified by multiplex PCR.

Odense University Hospital

Genotype

Variant alleles of DPYD: *2A; rs3918290 (c.1905+1G>A), *13; rs55886062 (c.1679T>G), rs67376798 (c.2846A>T) and HapB3 rs56038477 (c.1236G>A) were genotyped using the LAMP Human DPD deficiency KIT on a LC-GENIE III instrument (LaCAR MDx Technologies, Ougrée, Belgium). Genotyping was performed directly on EDTA-stabilized whole blood. After amplification melting curve analysis was used to determine the genotypes of each variant using the LC-GENIE III software.

Phenotype

A subset of the patients (n=206) from Odense University Hospital were analyzed as part of a research project after patients had received FP treatment. The blood was collected before initiation of FP treatment and stored in a biobank before analysis. These patients were *DPYD*-genotyped before their first dose of FP.

Plasma concentrations were measured by Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS). In brief, 300 µl plasma and 20 µl Internal Standard (0.5 µg/ml, 13C14N2-Uracil (Toronto Reseach Chemicals)) was mixed and 900 µl cold acetonitril was added. After centrifugation the clear supernatant was collected and evaporated under dry Nitrogen, The sample was reconstituated in 100 µl 0.1 % Formic Acid (aq). 25 µl was injected on the chromatographic system (TLX-1 Vanquish (Thermo Scientific)) The analytic column was a Phenomenex Kinetex PS18 2.6µm, 150x3.0 mm. Uracil and Internal Standard was detected using a TSQ Altis (Thermo Scientific) opparated in positive elektospray. The analysis was calibrated by in-house prepared calibrators and the relative standard deviation is below 10 %.

University Hospital of Southern Denmark, Lillebaelt Hospital

Genotype

Genomic DNA was isolated from EDTA-stabilized whole blood samples using the QIAsymphony SP-system (Qiagen). Variant alleles of DPYD: *2A; rs3918290 (c.1905+1G>A), *13; rs55886062 (c.1679T>G), rs67376798 (c.2846A>T) and HapB3 rs56038477 (c.1236G>A) were identified by genotyping based on TaqMan assays: C_30633851_20, C_11985548_10, AN9HXY3 and C_25596099_30 (Applied Biosystems/Thermo Fisher Scientific). Real-time PCR was performed using QuantStudio 12K Flex equipment (Applied Biosystems/Thermo Fisher Scientific), and the accompanying software v.1.3 was used for data analysis.

Phenotype

Plasma uracil levels were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Samples were precipitated with acetonitrile, and phospholipids removed using an Ostro 96-well plate from Waters. Then the samples was evaporated and reconstituted in 0.1% formic acid, before analysis on LC-MS/MS using a Phenomenex Kinetex 2,6 µm, PS C18 100A, 150*3 mm column. The analysis was calibrated by in-house prepared calibrators and the relative standard deviation is below 10 %. The LOQ (limit of quantification) was set at 5 µg/L because of harmonization with other danish laboratories under the premise that it is not clinically relevant to

quantify lower concentrations. However, it was validated with repeated analysis of a plasma pool with a mean concentration of 3.3 µg/L determined with a CV% of less than 10%.

Aalborg University Hospital

Genotype

Genomic DNA was isolated from whole blood samples using the Qiasymphony system (Qiagen). Variant alleles of *DPYD*: *2A; rs3918290 (c.1905+1G>A), *13; rs55886062 (c.1679T>G), rs67376798 (c.2846A>T) and the HapB3 haplotype variant alleles: rs75017182 (c.1129-5923C>G), rs56038477 (c.1236G>A), and rs56276561 (c.483+18G>A) were identified by multiplex PCR, using the commercial kit Elucigene *DPYD* (Yourgene Health, UK). The amplicon sizing was sorted by capillary electrophoresis on a genetic analyzer (3130xl Genetic Analyzer, Applied Biosystem). GeneMarker v.2.6.4 software (SoftGenetics) was used to visualize, process, and generate reports following provided kit instructions by Elucigen.

Aarhus University Hospital

Genotype

Genotypes were determined using the LAMP Human DPD deficiency KIT (LaCAR MDx Technologies, Ougrée, Belgium) assaying the four *DPYD* alleles: NM_000110.4:c.1905+1G>A, rs3918290; NM_000110.4:c.1679T>G, rs55886062; NM_000110.4:c.2846A>T, rs67376798 and NM_000110.4:c.1236G>A, rs56038477. Genotyping was performed directly on EDTA stabilised whole blood. Thermocycling and data collection was performed in cobas z480 Real Time PCR cyclers (Roche, Hvidovre, Denmark). Melting Curve analysis was used to determine the genotypes of each variant using the User Defined Functionality of the software. Genotypes were scored by two different individuals.

Phenotype

Plasma concentrations of uracil were measured by High Performance Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS). The liquid chromatography was carried out on the Agilent 1290 Infinity Series system (Agilent, Denmark) and mass spectrometric detection was carried out on the Agilent 6490 Triple Quad mass spectrometer (Agilent, Denmark), which was equipped with an electrospray ionization source. Analytical separation was performed on a Kinetex C18 HPLC column (3.0 x 150 mm, 2.6 µm) (Phenomenex, Denmark) at a temperature of 30 °C controlled by a column heater. The limit of detection was 5 ng/ml, limit

of quantification (LOQ) was 12,8 ng/ml and the total imprecisions (CV) were within the acceptance criteria (< 15%; <20% at LOQ).

Zealand University Hospital

Genotype

Test for DPYD-variants from January to April 20, 2021:

Genomic DNA was isolated from whole blood samples using the Qiasymphony system (Qiagen). Variant alleles of DPYD: *2A; rs3918290 (c.1905+1G>A), *13; rs55886062 (c.1679T>G), rs67376798 (c.2846A>T) and the HapB3 haplotype variant alleles: rs75017182 (c.1129-5923C>G)/rs56038477 (c.1236G>A) were identified by PCR and sequencing. The amplicon sequencing was performed by capillary electrophoresis on a genetic analyzer (ABI3500 Genetic Analyzer, Applied Biosystem). Mutation Surveyor v.4.0.9 software (SoftGenetics) was used to visualize, process, and generate reports.

The amplification primers of the DPYD specific variants:

Primer name	Primer sequences including M13 tales 5´->3´
DPYD_c.1679_F	tgtaaaacgacggccagtTGCTGTGTTGAAGTGAT
DPYD_c.1679_R	caggaaacagctatgaccGACATTTCTATATGACTTCAAT
DPYD_c.1905+1_F	tgtaaaacgacggccagtAATCATCCGGGGAACCACCT
DPYD_c.1905+1_R	caggaaacagctatgaccGCTGCTTCTGCCTCAGGTTTA
DPYD_c.2846_F	tgtaaaacgacggccagtCCCAAGTTAATATAATGCAACATTG
DPYD_c.2846_R	caggaaacagctatgaccCAAGAAGAAACATGTCTCATAGC
DPYD_c.1129-5923_F	tgtaaaacgacggccagtCAGACCAAATCATCGCATTACA
DPYD_c.1129-5923_R	caggaaacagctatgaccAGCAAACATGCCAACCTCT

Test for DPYD-variants from April 20 to December, 2021:

Variant alleles of DPYD: *2A; rs3918290 (c.1905+1G>A), *13; rs55886062 (c.1679T>G), rs67376798 (c.2846A>T) and the HapB3 haplotype variant alleles: rs75017182 (c.1129-5923C>G)/ rs56038477 (c.1236G>A)) were identified in genomic DNA from whole blood samples using the commercial CE/IVD kit LC-DPD4Mut-LP-24 (LaCAR, BE). The direct PCR reaction and melt curve analysis was performed on a genetic analyzer (LC-Genie III, LaCAR). Gen3-1440 software (LaCAR) was used to visualize, process, and generate reports following provided kit instructions by LaCAR.

Rigshospitalet

Genotype

DPYP-genotyping was performed directly on whole blood using the commercially available Lamp Human DPD Deficiency Kit (LaCar, Lièges, Belgium) for the variants *2A; rs3918290 (c.1905+1G>A), *13; rs55886062 (c.1679T>G, p.(Ile560Ser)), rs67376798 (c.2846A>T, p.(Asp949Val)) and rs56038477 (c.1236G>A, p.(Glu412Glu). In case of any discrepant results, PCR amplification followed by Sanger sequencing of the relevant variant was performed.

Phenotype

Plasma uracil was measured using a LC-MS/MS based method. Plasma was isolated and frozen within two hours after blood sampling. The samples were kept at 4 °C until the aliquot was frozen.

In a 96 well-plate, 75 µl plasma and 300 µl internal standard (50µg/L $^{13}C^{15}N_2$-Uracil (Toronto Research Chemicals) disolved in acetonitril) were add and shaken for 10 min. After centrifugation 300 µl of the supernatant were transferred to a new 96-well plate and vaporized under a nitrogen stream. The precipitate was dissolved in 200 µl of 95 % acetonitrile + 5 % 100 mM ammoniumacetate buffer, pH = 4.5. After shaking 10 min the samples were transferred to the LC-MS/MS system (Acquity-uPLC/Quattro Micro, Waters). The analytical column was a ZIC-HILIC, 3.5 µm 200 Å, sorbent, 100x2,1 mm (Merck) kept at 35 °C. The chromatography (flow rate 0,4 ml/L) consisted of a gradient started acetonitril with increasing proportion of 100 mM ammoniumacetate buffer, pH = 4.5.